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ON-COLUMN SILYLATION OF β-BLOCKING AGENTS APPLIED TO A BACK-FLUSHABLE PRECOLUMN CAPILLARY SYSTEM

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SUMMARY

Glass capillary gas chromatography with a packed back-flushable precolumn has been used for quantitative analyses of β-blocking agents. The compounds were converted into the corresponding O-trimethylsilyl derivatives by on-column derivatization using N,O-bis(trimethylsilyl)trifluoroacetamide.

The β-blocking agents were also analysed using a packed column. Pre-column silylation in reaction vials and on-column silylation were compared for both column methods. The concentration ranges investigated were 5–100 μg/ml for the back-flushable precolumn capillary system and 10–100 μg/ml for the packed column system.

The back-flushable precolumn capillary system was also applied to the analysis of plasma extracts.

INTRODUCTION

Extracts from natural products and biological materials containing compounds having a wide range of boiling points often require a time-consuming clean-up procedure before gas chromatographic (GC) analyses. Especially when working with glass capillary columns, it is important to protect the column from non-volatile and highly polar compounds which may reduce the efficiency of the analytical system. This can be achieved by incorporation of a short back-flushable precolumn. It is then possible to introduce into the main column only the compounds to be analyzed and back-flush out of the precolumn the part of the sample which is analytically of no interest. This system facilitates the sample clean-up procedure and the analysis time is reduced. The advantages and applications of the use of a back-flushable precolumn in combination with a capillary column have been described by Kaiser[1] and Schomburg[2].

The technique of direct derivatization by simultaneous injection of the sample and reagent has been applied to different drug analyses on both packed (on-column derivatization) and glass capillary columns (flash-heater derivatization)[3–5]. The purpose of the present investigation was to demonstrate the on-column derivatization technique combined with a back-flushable precolumn system and glass capillary

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column for quantitative analyses of drugs. β-Blocking agents were selected as model substances and the on-column silylation technique was applied to protect their functional groups. The same compounds were also analyzed using a packed column GC method without a precolumn. Both pre-column silylation in reaction vials at room temperature and on-column silylation were performed for both column methods. The linearity, reproducibility and the lower detection limits were evaluated and the results obtained from the different derivatization techniques were compared. The back-flushable precolumn capillary system was also applied to the analysis of plasma extracts.

The advantages and limitations of the system are discussed.

EXPERIMENTAL

Instrumentation

A Fractovap 2900 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector, a PC-IN 68/156 precolumn injector with an auxillary carrier gas inlet at the end of the precolumn and a Multiflow Control MFC 393 (Dani, Monza, Italy) was used. The precolumn (15 cm × 3 mm) was packed with 3% SE-30 on Supelcoport (80–100 mesh) and the packing length was 7 cm. A glass capillary (20 m × 0.25 mm I.D.) wall-coated open tubular (WCOT) CP Sil 5 column (Chrompack, Middelburg, The Netherlands) was used. The capillary column was mounted about 1 cm into the precolumn and 1 cm below the packing material. The injection port temperature was 275°C and the oven temperature program was as follows: 70°C at sample injection, then raised at 20°/min to 140°C or 160°C and then at 10°/min to the final temperature. Helium was used as the carrier gas and the flow-rate was adjusted to the following values: 3.5 ml/min through the capillary column, 55 ml/min through the outlet at the precolumn end (splitter) and 10 ml/min as septum flush. The two carrier gas inlets were adjusted to give the same flow-rate for the normal flow position and the back-flush position. The splitter was closed 2 sec after the injection, and when the last substances of interest had entered the capillary column the precolumn was back-flushed. The switching operation was programmed by means of the multiflow control module. The optimum time for switching was determined with standard solutions.

For the packed column analyses a Fractovap 2300 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector was used. The column (1 m × 2 mm) was packed with 3% SE-30 on Supelcoport (80–100 mesh). The injection port temperature was 275°C and the oven temperature was 130°C during injection; the temperature was then set at 200°C and programmed at 10°/min to 240°C. The flow-rate of the nitrogen carrier gas was 30 ml/min. A Spectra-Physics Autolab Minigrator was used for peak area measurements for both column methods.

GC–mass spectrometry (MS) was carried out using a Finnigan MAT mass spectrometer combined with a SE-54 fused-silica capillary column (30 m × 0.19 mm).

Drug standards and chemicals

The β-blocking agents were obtained as gifts from the following manufacturers: Alprenolol hydrochloride (Hässle, Mölndal, Sweden), oxprenolol hydrochloride (Ciba-Geigy, Basle, Switzerland), propranolol hydrochloride (I.C.I., Macclesfield, Great Britain), timolol maleate (MSD, Haarlem, The Netherlands) and pindolol base (Sandoz, Basle, Switzerland).

Eicosane was from Koch-Light (Colnbrook, Great Britain) and ampoules (1 ml) of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from Supelco (Bellefonte, PA, U.S.A.). Other reagents were of analytical grade obtained from E. Merck (Darmstadt, G.F.R.).

Stock standard solutions of the β-blocking agents and eicosane contained 1 mg/ml in ethyl acetate or dimethylformamide. They were diluted in ethyl acetate to the final concentration.

Calibration graphs and reproducibility tests

A calibration graph in the concentration range 5–100 μg/ml was constructed for propranolol using oxprenolol as internal standard (50 μg/ml) for the precolumn capillary system. The concentration range for the packed column analyses was 10–100 μg/ml. The on-column derivatization reaction was performed by injecting 1 μl test solution together with 2 μl of the derivatization reagent. Five assays on each solution were carried out and the regression lines and correlation coefficients were calculated.

For the reproducibility test, solutions containing 10 and 50 μg/ml were analyzed for both column methods. The mean and the relative standard deviation (R.S.D.) of ten assays were calculated.

RESULTS AND DISCUSSION

Derivatization studies and quantitative application

The effect obtained by employing the back-flushable precolumn capillary system at different switching programs is demonstrated in Fig. 1. Fig. 1a shows a mixture of the β-blocking agents after on-column silylation and back-flushing when all compounds had entered the main column. The chromatogram obtained in Fig. 1b is the result of a second injection of the same mixture with back-flushing 150 sec earlier. This figure shows that compounds 6, 5 and most of 4 have been back-flushed.

The injection port and precolumn temperatures have to be high enough to allow evaporation and quantitative derivatization of the sample. The initial capillary column temperature and its relationship to the boiling point of the solvent is also important for a good solvent effect and to obtain the best response. The effect of different column temperatures has been studied previously[5]. In this investigation a column temperature of 70°C for ethyl acetate (b.p. 77°C) as solvent yielded satisfactory results for the first four eluted compounds in Fig. 1a. However, a high boiling solvent such as dimethylformamide combined with a higher initial column temperature was more favourable for the last eluted compounds.

Formation of trimethylsilyl derivatives of the hydroxy groups after BSTFA injection together with the sample was demonstrated by GC–MS measurements. Only one peak from each compound could be detected. From this investigation it could also be concluded that the secondary amine group in the side-chain was not silylated.

In order to verify the back-flushable precolumn capillary system for quantitative application, a calibration graph in the concentration range 5–100 μg/ml was constructed for propranolol using oxprenolol as internal standard. The data obtained for the regression line and the correlation coefficient together with data from the reproducibility tests are acceptable for quantitative use (Table I).

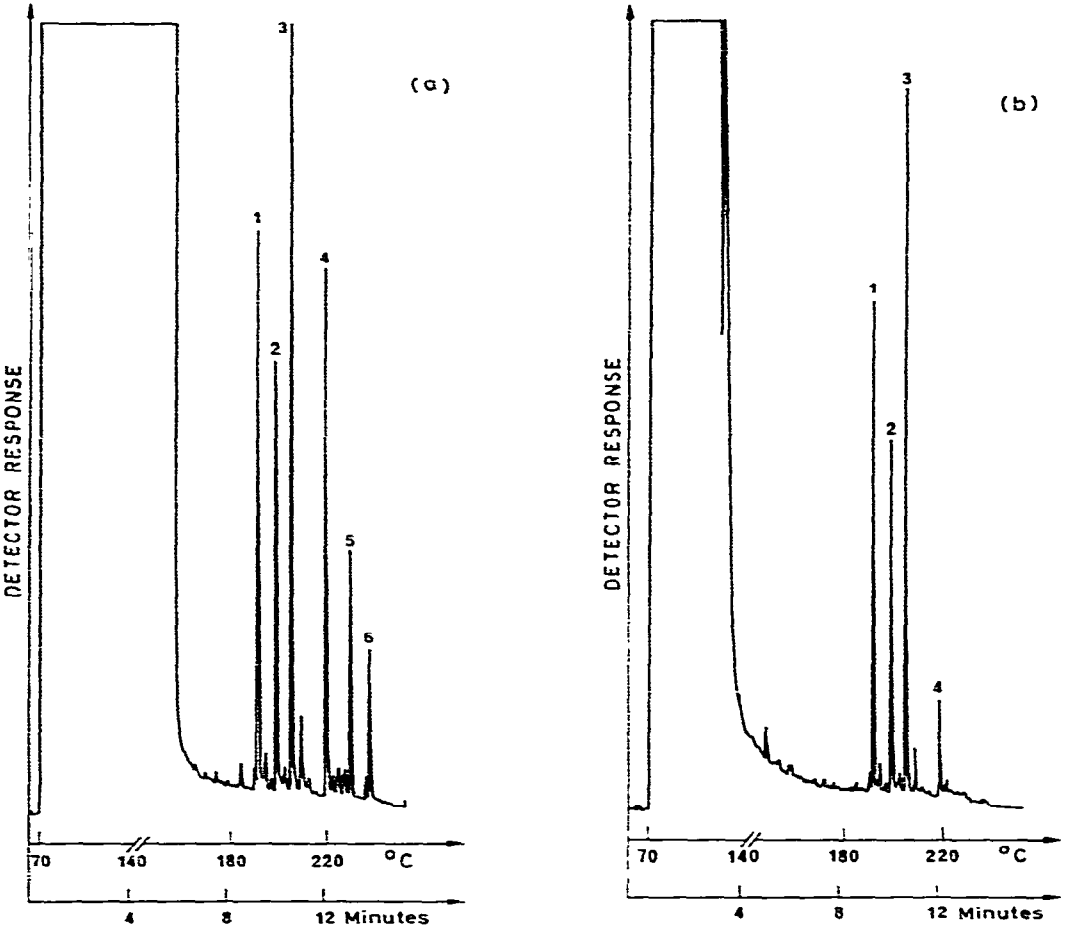

Fig. 1. Chromatograms obtained from the back-flushable precolumn capillary system at different switching programs: a, a test mixture containing 10 ng each of alprenolol (1), oxprenolol (2), eicosane (3), propranolol (4), timolol (5) and pindolol (6) after on-column silylation, the precolumn being back-flushed 300 sec after injection; b, is the result of a second injection of the same mixture with back-flushing 150 sec earlier. For other chromatographic conditions see text.

Comparison of on-column and pre-column derivatization in reaction vials was undertaken by treating different standard extracts with BSTFA and pyridine at room temperature, a method described previously for the silylation of timolol[6]. The results also given in Table I, from which it is concluded that no significant difference can be observed between the two methods. However, somewhat lower precision was obtained from the pre-column derivatization method. Also several extra peaks appeared in the chromatograms which interfered with the sample peaks at low concentration.

Comparison with the packed column system was also made. The calibration graph (10–100 μg/ml) together with data from the reproducibility tests, given in Table I, were in accordance with the results obtained from the back-flushable precolumn capillary system. In the packed column analyses, the values from the precolumn

TABLE I

DATA FROM CALIBRATION GRAPHS AND REPRODUCIBILITY TESTS USING PROPANOLOL AS TEST SUBSTANCE AND OXPRENOLOL AS INTERNAL STANDARD ON DIFFERENT COLUMN SYSTEMS

Column system	*Calibration graph*	*Correlation coefficient*	*Concentration (μg/ml)*	*On-column*		*Pre-column*	
				Peak area ratio	*R.S.D. (%)*	*Peak area ratio*	*R.S.D. (%)*
Capillary column (5–100 μg/ml)	$y = 0.020x + 0.014$	0.998	100	2.13	4.3	2.19	5.6
			50	1.03	4.2	1.06	5.5
			25	0.50	5.0	0.51	5.8
			10	0.19	5.0	0.19	7.5
Packed column (10–100 μg/ml)	$y = 0.023x - 0.056$	0.998	100	2.17	2.8	2.14	4.3
			50	1.05	3.1	1.07	4.6
			25	0.51	2.2	0.52	3.5
			10	0.18	1.5	0.18	6.9

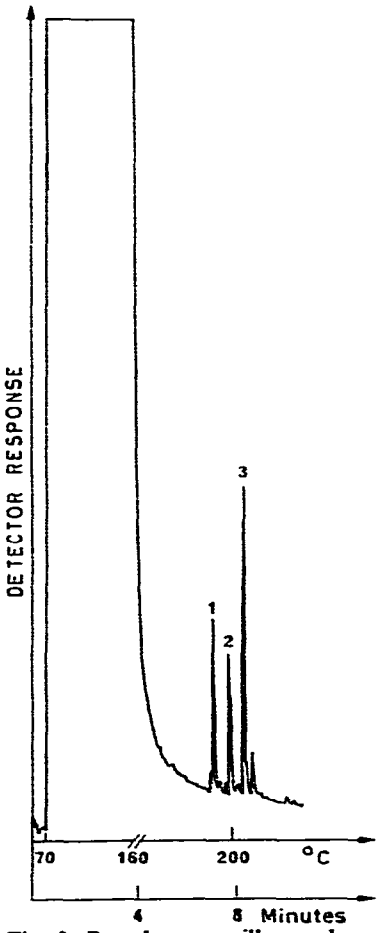

Fig. 2. Precolumn–capillary column chromatogram obtained after on-column silylation of a mixture containing 2 ng each of alprenolol (1) and oxprenolol (2) and 5 ng of eicosane (3). The precolumn was back-flushed 120 sec after the injection. For chromatographic conditions see text.

derivatization at room temperature showed a somewhat higher spread compared to on-column derivatization. For both derivatization methods, better precision was obtained for the packed column application. Earlier work has also shown that the capillary column system is more sensitive to the different parameters such as the solvent, the initial column temperature and the injection technique as compared to a packed column system[7].

The limit of detection on the packed column was about 5 ng while 500 pg could be detected on the back-flushable precolumn capillary system. Excess of reagent and by-products caused interferences when lower sample sizes were injected. Fig. 2 shows the chromatogram obtained upon injection of 2 ng alprenolol and oxprenolol and 5 ng eicosane.

Plasma analysis

The precolumn capillary system was applied to plasma sample analyses. 1-ml volumes of spiked plasma samples containing alprenolol and oxprenolol were extracted according to a modification of an earlier method[8]. Fig. 3 shows a chromato-

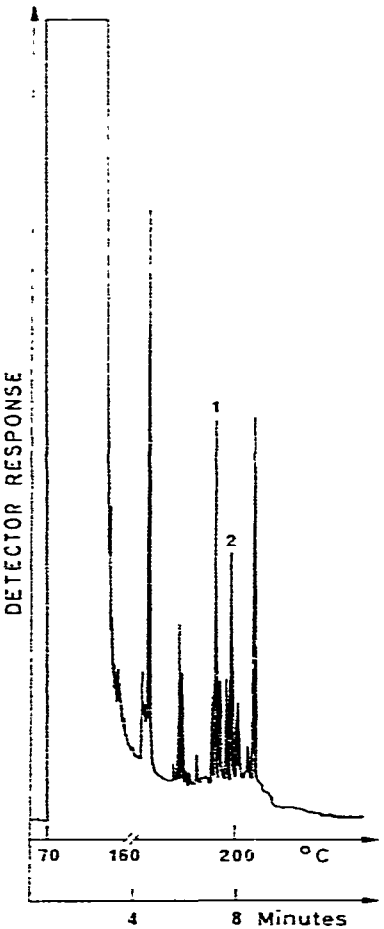

Fig. 3. Chromatogram obtained after on-column silylation of a plasma extract containing alprenolol (1) and oxprenolol (2) (200 ng/ml plasma). The precolumn was back-flushed 85 sec after the injection. For chromatographic conditions see text.

gram obtained from a plasma extract containing 200 ng/ml plasma of each compound. The precolumn was maintained in the back-flush position after the last peak in the chromatogram. However, for analyses at the lower therapeutic concentration levels, a specific detector and derivatization agents must be employed. Extra peaks from plasma with t_R values similar to those of the sample peaks will interfere at lower concentration and the sensitivity setting cannot be completely utilized. Studies with specific detectors connected to the back-flushable precolumn system are planned for analyses of therapeutic plasma samples.

CONCLUSIONS

By adding a precolumn to the capillary column system, overloading can be overcome to a large extent. This column can be removed and repacked without touching the capillary column. From this study it can be concluded that the on-column derivatization technique is applicable to the back-flushable precolumn capillary system for quantitative analyses. The data are in accordance with the results obtained from pre-column derivatization at room temperature. Agreement with packed column analyses was also demonstrated, although the capillary column method showed somewhat lower reproducibility. This observation is consistent with a previously published comparison of capillary and packed column methods[7]. The most important advantage of the present system is the saving in analysis time for plasma extracts and natural products, as it is possible to simplify the preliminary treatment. By appropriate adjustment of the rate and time of splitting and back-flushing, it is possible to protect the capillary column from low-boiling solvents and substances of low volatility. The appearance of ghost peaks is eliminated and the lifetime of the capillary column is lengthened.

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